Differential Activation of Protein Kinase C Isozymes by Phorbol Ester and Collagen in Human Skin Microvascular Endothelial Cells

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Human dermal microvascular endothelial cells participate in activities including inflammation, wound healing, and angiogenesis (neovascularization). Two stages of angiogenesis can be mimicked *in vitro* by two models of cultured foreskin human dermal microvascular endothelial cells: the differentiation of epithelioid endothelial cells to spindle-shaped mesenchymal-like cells induced by phorbol ester treatment; and the formation of vascular channels induced by exposing the luminal surface of endothelial cell monolayers to type I collagen gels. The mechanisms underlying these two processes, however, are largely unknown. Protein kinase C isozymes, which are activated by phorbol esters, are important mediators in the angiogenic process. In the current work, we identified the protein kinase C isozymes present in human dermal microvascular endothelial cells and determined which of the isozymes are activated in response to phorbol ester or to collagen treatments. Using western blot analysis, we found that microvascular endothelial cells contain at least six protein kinase C isozymes (α, β, δ, ε, ζ, η). Immunocytochemical studies demonstrated that the isozymes are located in distinct cellular compartments and that following treatment with phorbol 12-myristate 13-acetate or with a collagen gel overlay, most isozymes (protein kinase C α, βI, βII, δ, ε, η) translocated to different parts of the cell. Moreover, for two of these isozymes (βII and η), the localization differs after phorbol 12-myristate 13-acetate treatment as compared with collagen treatment. These results demonstrate that agents that mimic two stages in the angiogenic process *in vitro* initiate diverse changes in the subcellular localization of specific protein kinase C isozymes and suggest a role for different isozymes in this process. ***Key words: human dermal microvascular endothelial cells/angiogenesis. J Invest Dermatol 107:248–252, 1996***

One of the first steps in angiogenesis is the conversion of microvascular endothelial cells from the typical epithelioid configuration to mesenchymal-like, spindle-shaped cells (Karasek, 1991). This step precedes the migration of human dermal microvascular endothelial cells (HDMEC) and formation of vascular channels.

Earlier studies demonstrated that both large vessel cells and microvascular cells undergo a rapid change from an epithelioid to a spindle-shaped morphology when treated with a phorbol ester or cytokines (Antonov *et al*, 1986; Lipton *et al*, 1991). There is also a striking change in morphology and the growth pattern of cells when the luminal surface of a confluent culture is covered with a collagen gel: HDMEC differentiate within 4 h to form vascular channels (Kramer *et al*, 1987). The biochemical mechanisms underlying the intracellular signaling events involved in this differentiation are unknown.

Protein kinase C (PKC) is a key enzyme in signal transduction regulating cell growth (Olson *et al*, 1993), cell differentiation (Saxon *et al*, 1994), and gene expression (Blackshear *et al*, 1988). The activation of PKC by β-phorbol 12-myristate 13-acetate (PMA) in human umbilical vein endothelial cells has been shown to be required for the differentiation of these cells to capillary-like structures when cultured in the presence of Matrigel and in the absence of serum (Kinsella *et al*, 1992). In addition, PKC appears to regulate the expression and rearrangement of adhesion molecules on endothelial cells (Sung *et al*, 1994) and the retraction of large blood vessel endothelial cells by lipoxygenase metabolites (Tang *et al*, 1993).

PKC constitutes a family (α, βI, βII, γ, δ, ε, ζ, η, θ, λ, and μ) of related isozymes (Nishizuka, 1988, 1989). They consist of a single polypeptide with an amino-terminal regulatory domain and a carboxy-terminal catalytic domain (Kikkawa *et al*, 1987; Ono *et al*, 1988). Differences in tissue distribution, subcellular localization, and translocation following activation have also been demonstrated (Mochly-Rosen *et al*, 1990; Disatnik *et al*, 1994). Previous studies regarding the expression of PKC isozymes in bovine aortic endothelial cells (Rosales *et al*, 1992), and human umbilical vein endothelial cells (Deisher *et al*. 1993) have been performed using anti-α, -β, and -γ PKC antibodies. These studies in large blood vessel cells also demonstrated that individual isozymes have different translocation patterns upon activation. Individual isozymes are likely to play a distinct role in signal transduction and therefore influence different cell functions.

We used isozyme-specific PKC antibodies to identify the PKC isozymes present in human dermal microvascular endothelial cells.

Manuscript received December 4, 1995; revised March 26, 1996; accepted for publication April 18, 1996.

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Abbreviations: HDMEC, human dermal microvascular endothelial cells; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PKC, protein kinase C.

0022-202X/96/$10.50

Since translocation of PKC upon stimulation is thought to indicate activation, we studied the translocation of PKC isozymes induced by either PMA treatment or collagen overlay on cultured HDMEC. We found that at least six PKC isozymes are present in HDMEC (α, β, δ, ϵ, ζ, η), and that the isozymes have distinct subcellular localizations. With only one exception (PKC ζ), all of the PKC isozymes translocated to a different subcellular location after treatment with PMA or collagen.

MATERIALS AND METHODS

Materials PKC isozyme-specific antibodies (anti-α, -β, and -γ PKC monoclonal antibodies and anti-δ, -ϵ, -ζ, and -η PKC polyclonal antibodies) used for immunocytochemistry were purchased from Research and Diagnostic Antibodies (Berkeley, CA). Polyclonal antibody anti-θ PKC was from Santa Cruz Biotechnology (Santa-Cruz, CA). Fluorescein-conjugated anti-rabbit IgG antibodies prepared in goat were from Organon Technika Corp. (West Chester, PA). For western blot analyses, anti-α, -β, and -γ PKC monoclonal antibodies were from Seikagaku America Inc (Rockville, MD), and anti-δ, -ϵ, and -ζ PKC polyclonal antibodies were from GIBCO BRL (Gaithersburg, MD). Anti-η PKC was obtained from Research and Diagnostic Antibodies (Berkeley, CA). Anti-θ PKC was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum to mouse IgG was from Organon Teknika Corp. (Durham, NC). Glass slides with eight chambers came from Nunc, Inc. (Naperville, IL). αPMA was from LC Laboratories (Woburn, MA). PMA was purchased from Sigma Chemical Co. (St. Louis, MO). Collagen was from Celtrix (Santa Clara, CA). [^{125}I]protein A was from ICN Pharmaceuticals, Inc. (Irvine, CA).

Cell Culture Human dermal microvascular endothelial cells were isolated from neonatal human foreskin as previously described (Karasek, 1989). Using gelatin-coated plastic flasks, cells were grown in a complete media containing modified Iscove's growth media supplemented with 8% newborn calf serum, 2% human prepartum maternal serum, 1.3×10^{-4} M dibutyryl cAMP, 3.3×10^{-5} M isobutyl methylxanthine, 1×10^{-4} M hypoxanthine, and 1.5×10^{-5} M thymidine. Cells were fed twice each week and were trypsinized using 0.1% trypsin and 1% ethylenediamine tetraacetic acid when cells became confluent. Second through fourth generations of HDMEC were routinely used in all experiments. Approximately 95% of cells expressed specific antigens in endothelial cells including factor VIII and platelet endothelial cell adhesion molecule. For PKC translocation studies, approximately 3000 cells per chamber were seeded on glass chamber slides coated with 1% of gelatin. Cells were fed twice each week with complete medium until they reached 95–100% confluency.

Western Blot Analysis Confluent cells cultured in 75-mm flasks were washed twice with phosphate-buffered saline (PBS) and scraped on ice into 1 ml of buffer containing 20 mM Tris(hydroxymethyl)aminomethane-HCl, pH 7.5, 2 mM ethylenediamine tetraacetic acid, 10 mM ethyleneglycol-bis(β-aminoethyl ether)-*N,N, N',N'*-tetraacetic acid, 20 μg aprotinin per ml, 20 μg leupeptin per ml, 20 μg soybean trypsin inhibitor per ml, and 10 μM phenylmethylsulfonyl fluoride. The cell suspension was passed through a 25-gauge needle five times, and samples (50 μg/lane) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electroblotting onto a nitrocellulose membrane. The membrane was subsequently incubated for 2 h in a blocking solution containing 5% dried milk in PBS containing 0.05% Tween 20. PKC isozyme levels were determined using anti-α, -β (which recognize both βI, βII PKC isozymes), or -γ PKC monoclonal antibodies diluted 1:1000 followed by 1-h incubation with rabbit anti-mouse antisera (1:1000), or anti-δ, -ϵ, -ζ, -η, or θ PKC polyclonal antibodies diluted 1:300. All membranes were then incubated with [^{125}I]protein A, and PKC immunoreactive bands were detected by autoradiography.

Immunolocalization of PKC Isozymes Cells grown in gelatin-coated chamber slides were washed three times (1 h each) with serum-free complete medium to eliminate serum; cells were then treated with 0.1 μg PMA per ml (162 nM) for 10 min or with collagen overlay (containing 50% vol/vol Vitrogen 100, 50% vol/vol twice concentrated Dulbecco's modified Eagle's medium, and 1% vol/vol 0.5 N NaOH, final pH 7.4) for 30 min to allow complete gelation of collagen (Lipton *et al*, 1991). Negative controls were treated with 4-αPMA, a biologically inactive phorbol ester. After treatment, cells were washed once with PBS, fixed for 3 min in a 1:1 mixture of acetone-methanol at −20°C, and subsequently washed twice with PBS. Cells were then incubated at room temperature for 1 h with 1% normal goat serum in PBS containing 0.1% Triton X100 in order to minimize nonspecific binding. Cells were then incubated overnight at 4°C with the PKC isozyme-specific antibodies diluted 1:100 in PBS containing 0.1% Triton X100 and 2% bovine serum albumin. Cells were washed three times with PBS containing 0.1% Triton X100 and incubated for 2 h with fluorescein-conjugated anti-rabbit IgG antibody diluted 1:1000 in PBS containing 0.1% Triton X100. After three washes with PBS, slides were mounted using Miowiol 4–88 (Calbiochem, La Jolla, CA) as described previously (Disatnik *et al*, 1994). A Zeiss IM35 microscope with a 40 × water immersion objective (Carl Zeiss, Inc., Thornwood, NY) was used to record images on Kodak Tri-X pan film with an exposure time of 45 s.

Specificity of the staining obtained using different anti-PKC isozyme antibodies was determined by pre-absorption of the α, βI, βII, δ, ϵ, ζ, or η antibodies (1:100) with their immunizing peptides (1 μg/μl) for 4 h at 4°C (Disatnik *et al*, 1994). No specific staining was found after pre-absorption or in cells stained in the absence of the PKC antibodies (data not shown).

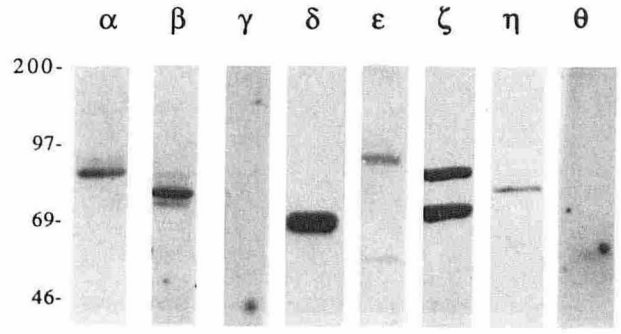

Figure 1. Immunoblot analysis of different PKC isozymes (PKC α, β, γ, δ, ϵ, ζ, η and θ) in HDMEC. Total proteins (50 μg per lane) from HDMEC were transferred to a nitrocellulose membrane following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% gel). The molecular mass markers were myosin (200 kDa), phosphorylase (97 kDa), bovine serum (69 kDa), and ovalbumin (45 kDa).

RESULTS

Identification of PKC Isozymes Present in Human Dermal Microvascular Endothelial Cells PKC isozyme expression in human dermal microvascular endothelial cells was examined by western blot analysis using isozyme-specific anti-α, -β, and -γ PKC monoclonal antibodies or anti-δ, -ϵ, -ζ, -η, and -θ PKC polyclonal antibodies. γ and θ PKC isozymes were not found in HDMEC extracts **(Fig 1)**. In contrast to these two isozymes, immunoreactive bands of six PKC isozymes, α, β, δ, ϵ, ζ, and η, were clearly detected **(Fig 1)**. Isozymes α, δ, and η had apparent molecular masses of 82, 72, and 80 kDa, respectively. PKC β was recognized as a protein with doublet bands of 76 and 78 kDa, whereas PKC ζ was detected as 75- and 82-kDa bands. A PKC ϵ immunoreactive band was detected at about 92 kDa. A minor band of 56 kDa of ϵ PKC immunoreactivity was also found.

Localization of PKC Isozymes in Human Dermal Microvascular Endothelial Cells To determine PKC isozyme localization in HDMEC, we performed immunofluorescence analysis using isozyme-specific antibodies as previously reported (Mochly-Rosen *et al*, 1990; Disatnik *et al*, 1994). This technique provides information regarding subcellular localization and redistribution of different isozymes at the level of individual cells. To verify the specificity of each isozyme, immunofluorescence staining was tested by using PKC isozyme-specific antibodies preabsorbed with their respective immunizing peptides. After preabsorption, no staining was observed (data not shown). These results are similar to those observed in cardiac myocytes (Disatnik *et al*, 1994).

When staining of nonstimulated HDMEC was examined using the anti-γ and anti-θ PKC antibodies, no staining was detected (data not shown). These findings confirm the western blot data in which the γ and θ PKC immunoreactivities were absent.

The immunoreactivity of αPKC appeared as diffuse staining throughout control (untreated) cells **(Fig 2*A*)**. Some nuclear staining was also observed. After treatment with PMA or collagen overlay, similar results were obtained, as shown in **Fig 2*B*** and **2*C***.

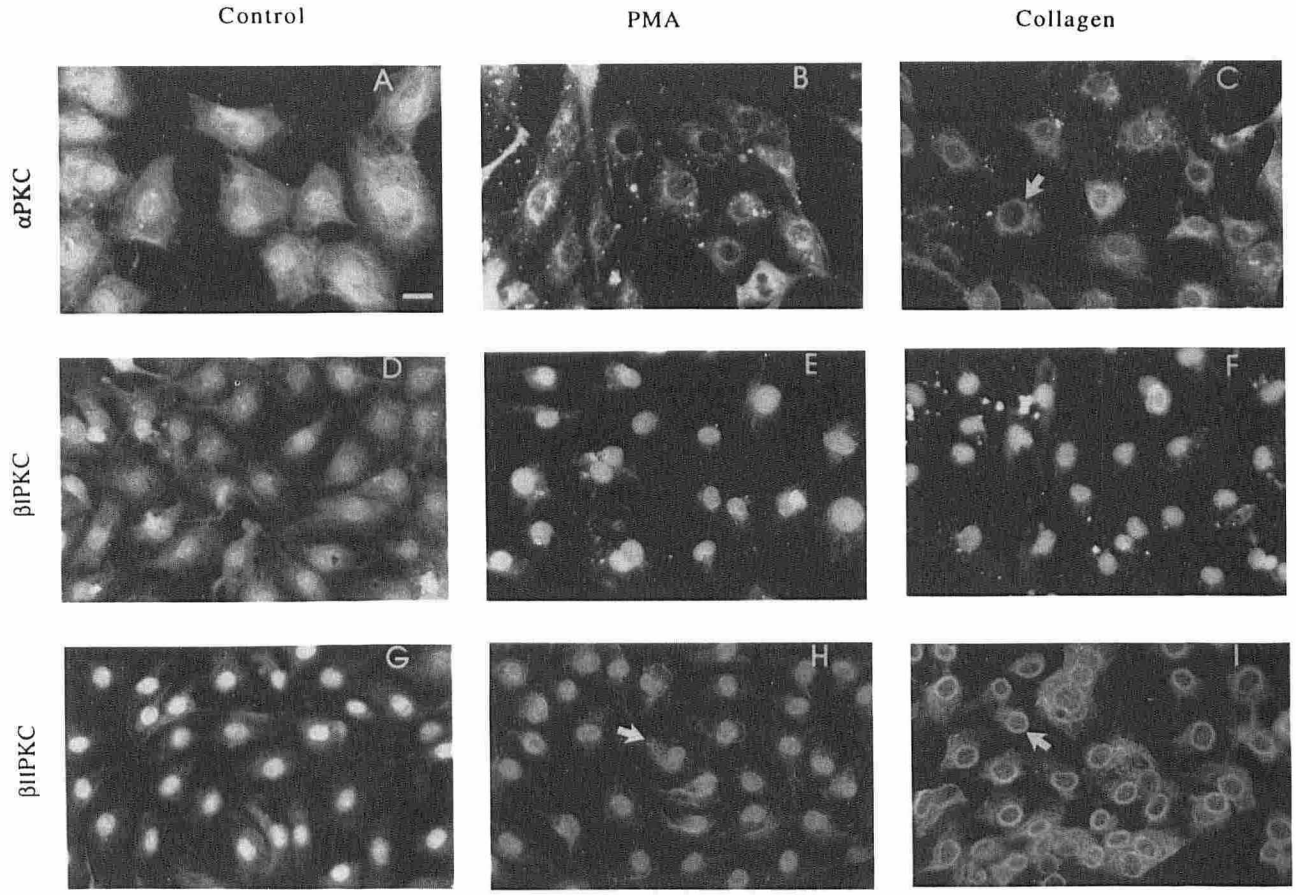

Figure 2. Immunofluorescence localization of α, βI, and βII PKC in HDMEC. Cells were fixed and stained with isozyme-specific antibodies as described in *Materials and Methods*. *A*, *D*, and *G*, nonstimulated cell; *B*, *E*, and *H*, PMA-treated cells (0.1 μg per ml, 10 min); *C*, *F*, and *I*, cells overlayed with collagen for 30 min. *Scale bar*, 5 μm. The ➪ in *C* indicates the staining at the perinuclear region. The ➪ in *H* indicates the filamentous staining.

Cytosolic staining and especially nuclear staining were reduced, and a peri-nuclear staining pattern appeared (**Fig 2*C***, *arrow*).

Anti-βI staining was fibrillar in the cytoplasm and diffuse in the nucleus in control cells (**Fig 2*D***). The intranuclear staining increased and cytoplasmic staining decreased after activation induced either by PMA or by collagen overlay (**Fig 2*E*, 2*F***). The βII PKC immunostaining was predominantly located in the intranuclear region of the control cells (**Fig 2*G***). After treatment with PMA, the intranuclear staining decreased and the filamentous staining in the cytoplasmic region was more intense than that found in untreated cells (**Fig 2*H***, *arrow*). The translocation of βII PKC was more pronounced when cells were treated with collagen. The immunostaining of the nucleus observed in control cells (**Fig 2*G***) was replaced with intense perinuclear staining (see ring-like structure in **Fig 2*I***, *arrow*). In addition, filamentous staining was also observed (**Fig 2*I***). Therefore, the localization of βII PKC following collagen and PMA treatments differed.

When HDMEC were examined using the anti-δ PKC antibody, the immunolocalization of δPKC was primarily cytosolic and fibrillar (**Fig 3*A***). After exposure to PMA (**Fig 3*B***) or collagen (**Fig 3*C***), the filamentous staining was more apparent in both situations (see *arrows* in **Fig 3*B*** and **3*C***). These staining patterns were similar to the ones obtained with anti-ζ PKC antibodies in either nonstimulated (**Fig 3*D***) or stimulated cells (**Fig 3*E*, 3*F***), indicating that there was no translocation of ζ PKC following either treatment.

The staining of control cells using anti-ϵ PKC antibody was diffuse in the cytoplasm and dotted in the periphery of the cells (**Fig 4*A***). In one half of the cultures, more intranuclear staining was observed, and the staining at the cell periphery was continuous (**Fig 4*a***, *small arrow*) rather than dotted (**Fig 4*A***). This pronounced peripheral staining pattern (**Fig 4*A*, 4*a***, *arrows*) was unique for ϵ PKC and disappeared following treatment with either PMA or collagen, whereas the cytosolic staining was only slightly decreased (**Fig 4*B*** and **4*C***). In addition, perinuclear staining was more apparent in cells stimulated with PMA or collagen.

The staining of control cells using anti-η PKC antibody was localized in the cytoplasm as well as in the nuclear region. After treatment with PMA (**Fig 4*E***), the nuclear staining was reduced and perinuclear staining appeared. This perinuclear staining was not observed, however, in collagen-treated cells (**Fig 4*F***), and the overall staining of collagen-treated cells resembled that of control cells (**Fig 4*D***). Therefore, η PKC translocated following PMA treatment but not following collagen overlay.

We also carried out immunofluorescence analysis of cells treated with 4-αPMA, the biologically inactive derivative of PMA. For each isozyme, no redistribution was observed following treatment with 4-αPMA (0.1 μg per ml for 10 min); the immunostaining of control cells and cells treated with 4-αPMA was identical (data not shown).

Table I summarizes the results obtained by immunofluorescence analysis of the seven isozymes found in HDMEC. The data show that PKC isozymes are located in distinct cellular compartments before and after activation with PMA and collagen.

DISCUSSION

A link between angiogenesis and PKC activation has been suggested (Ingber *et al*, 1990). Using the chick chorioallantoic membrane, Tsopanoglou *et al* (1993) demonstrated that PKC inhibitors can reverse the angiogenic effects induced by PMA. PKC may also play an important role in the functions of a number of skin cell types

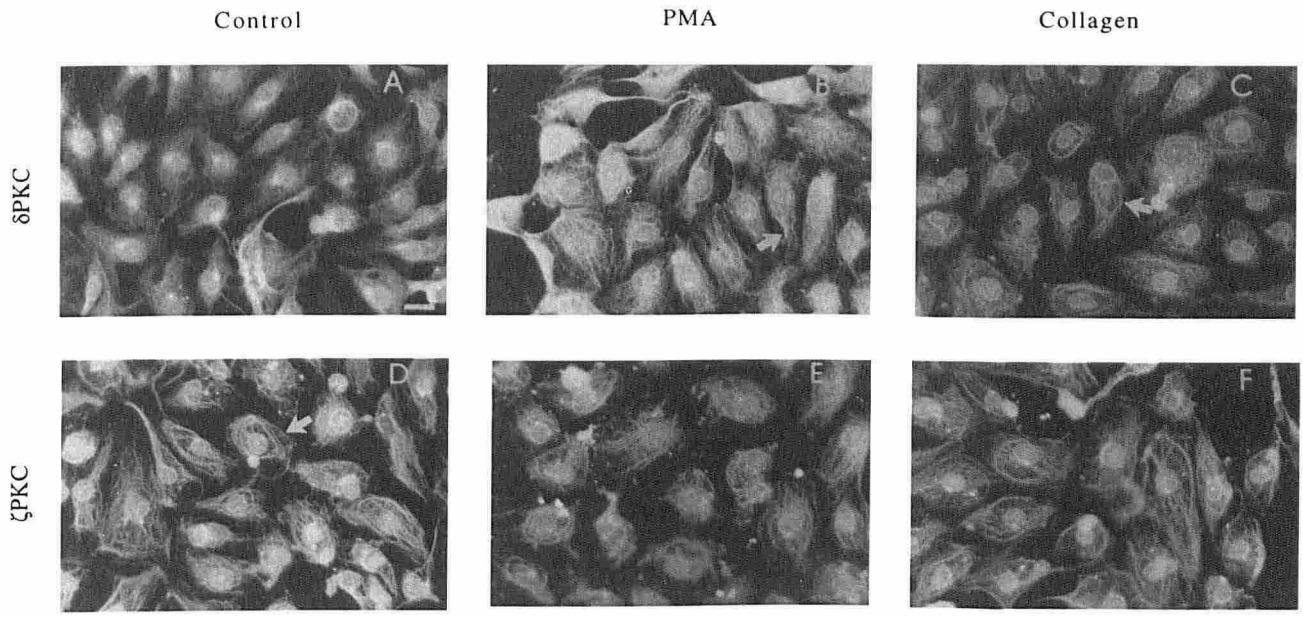

Figure 3. Immunofluorescence localization of PKC δ and ζ in HDMEC. Cells were fixed and stained with isozyme-specific antibodies as described in *Materials and Methods*. *A* and *D*, nonstimulated cells; *B* and *E*, PMA-treated cells (0.1 μg per ml, 10 min); *C* and *F*, cells overlayed with collagen for 30 min. *Scale bar*, 5 μm. The ➪ in *C* and *D* indicate the filamentous staining.

including the keratinocyte (O'Driscoll *et al*, 1994), and the microvascular endothelial cell (Davis *et al*, 1993). In human umbilical vein endothelial cells, vitronectin, one of the cell adhesion molecules implicated in the process of capillary-like structure formation, appears to be a substrate for PKC (Davis *et al*, 1993).

In the current study, we used two independent methods to show that multiple PKC isozymes (α, βI, βII, δ, ε, ζ, and η) are present in human dermal microvascular endothelial cells. The data obtained with isozyme-specific antisera, both by western blot analysis **(Fig 1)** and by immunofluorescence studies (data not shown), indicated that isozymes γ and θ were undetectable even under a variety of primary and secondary antibody dilutions. It is unlikely that the negative signals for both isozymes in western blot analysis and in immunofluorescence studies reflect a weak specificity for these two antibodies rather than a true absence of these isozymes at the protein level in HDMEC, because the same antibodies readily detected γ PKC in rat brain tissue (not shown) and in human T cells (Smith and Mochly Rosen, unpublished). The lack of γ and θ isozymes in HDMEC is in agreement with other studies indicating that the PKC γ isozyme is a brain-specific form (Nishizuka, 1988; Ono *et al*, 1988), it cannot be detected in bovine aortic endothelial cells (Rosales *et al*, 1992), and that PKC θ was predominantly expressed in hematopoetic cells (Baier *et al*, 1993).

It was not surprising to find that isozyme ζ failed to respond to PMA stimulation. Other studies (Ono *et al*, 1989; Nakanish and Exton, 1992) have suggested that ζ PKC has several distinguishing

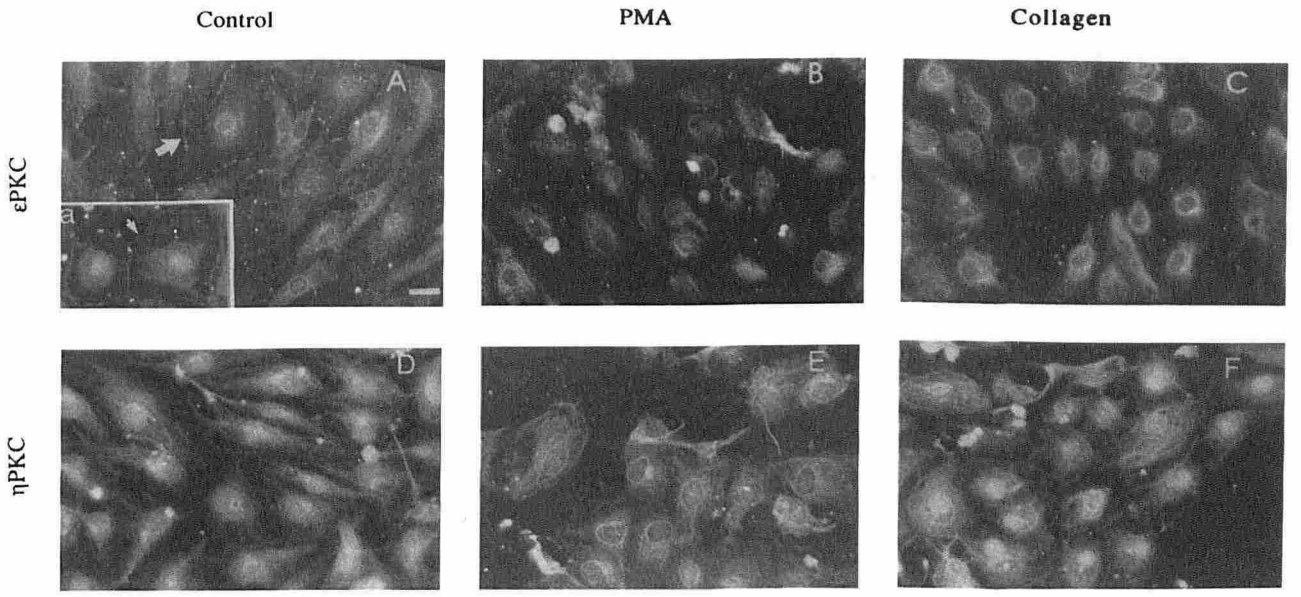

Figure 4. Immunofluorescence localization of PKC ε and η in HDMEC. Cells were fixed and stained with isozyme-specific antibodies as described in *Materials and Methods*. *A*, *a*, and *D*, nonstimulated cells; *B* and *E*, PMA-treated cells (0.1 μg per ml, 10 min); *C* and *F*, cells overlayed with collagen for 30 min. *Scale bar*, 5 μm. The ➪ in *A* and *a* indicate the anti-ε PKC staining at the cell periphery.

Table I. Localization of PKC Isozymes Before and After Activation with Phorbol Ester (PMA) and Collagen

Treatment	Control	PMA	Collagen
αPKC	Diffuse cytosolic and nuclear	Perinuclear	Perinuclear
βIPKC	Cytosolic fibrillar and some intranuclear	Intranuclear	Intranuclear
βIIPKC	Faint cytosolic and intranuclear	Filamentous and intranuclear	Some filamentous and mainly perinuclear
δPKC	Diffuse filamentous	Filamentous	Filamentous
εPKC	Cell periphery and perinuclear or intranuclear	Mainly perinuclear	Mainly perinuclear
ζPKC	Filamentous	Filamentous	Filamentous
ηPKC	Intranuclear and faint fibrillar	Perinuclear and fibrillar	Intranuclear and fibrillar

characteristics compared with other isozymes. First, ζ PKC contains only one cysteine-rich zinc-finger-like sequence whereas other PKC isozymes have two repeats of this sequence. Second, ζ PKC does not bind phorbol esters or diacylglycerol (Ono *et al*, 1989). In our study, ζ PKC did not respond to either PMA or collagen stimulation, suggesting that ζ PKC is probably not involved in HDMEC differentiation induced by PMA or collagen.

The immunofluoresence studies demonstrated that the localization of the isozymes following stimulation with PMA or collagen differed from that of untreated cells. The translocation of PKC has been noted in a number of cell types including bovine aortic endothelial cells (Rosales *et al*, 1992) and rat cardiac myocytes (Mochly-Rosen *et al*, 1990; Disatnik *et al*, 1994). As has been previously suggested (Mochly-Rosen, 1995), it is likely that the differentially localized isozymes phosphorylate nearby substrates. Further studies to identify isozyme-unique substrates at each of these isozyme sites are underway.

Although βI and βII PKC are derived from alternative splicing of the same mRNA (Ono *et al*, 1986) and differ in their C-terminal sequence by only 50 amino acid residues (Kubo *et al*, 1987), they are differentially localized in HDMEC. The immunostaining patterns of βI and βII PKC isozymes in HDMEC differ from each other before and after activation (**Fig 2*D*, *E*,** and ***F versus G*, *H*,** and ***I*).** These results suggest that the C-terminal sequence is essential for the localization of the activated isozymes and probably also the resulting function of βI and βII PKC in HDMEC. A similar difference in βI and βII PKC translocation was also observed in primary cardiac myocytes when stimulated with PMA or norepinephrine (Disatnik *et al*, 1994).

Our studies also demonstrate differential responses of the isozymes to PMA and collagen. The differences in localization of βII and η PKC isozymes in response to PMA or collagen treatments are of particular interest. They may provide a mechanism to explain why collagen treatment induces channel formation of endothelial cells whereas PMA treatment induces the conversion of epithelioid endothelial cells to spindle-shaped mesenchymal-like cells.

Differences in both the activation and translocation of PKC isozymes by phorbol esters and collagen gels, and the major differences in the induction of new vessels by these agents, suggest an involvement of different protein receptors for PKC isozymes. The development of specific translocation inhibitors of PKC isozymes may provide a new pharmacologic approach to the control of angiogenesis (Mochly-Rosen, 1995).

This work was supported by United States Public Health Service Grants 5ROI HL 484 95 and AR 41045 to M. A. Karasek and by HL43380 to D. Mochly-Rosen.

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